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Increased anti-HIV-1 activity of CD4 CDR3-related synthetic peptides by scrambling and further structural modifications, including D-isomerization and dimerization

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We recently showed that S1, a sequence-scrambled form of CD4 CDR3-related synthetic peptide, has more potent inhibitory activities on HIV-1 replication and HIV-1-induced syncytium formation than the original form. In this study, a series of derivatives of S1 were synthesized and their anti-HIV-1 activities were evaluated. A D-isomer was as potent as S1, and a homodimer was 10- to 18-fold more potent than S1. The increased antiviral activity of the dimer peptide was related to α-helix formation, as detected by circular dichroism.

HIV-1; CD4; Synthetic peptide; Antiviral activity; Circular dichroism

1. INTRODUCTION

The binding of human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein, gp120, to the cellular receptor CD4 is the first step in the infection of $CD4^+$ cells by HIV-1 [1–3]. The CD4 binding causes conformational changes in gp120 and leads to the dissociation of gp120 from the virus [4–6]. Some synthetic peptides derived from the CD4 region, corresponding to the immunoglobulin third complementarity-determining region (CDR3) or its adjacent region, which show inhibitory effects on HIV-1 infection and HIV-1-induced syncytium formation [7–9], also induce the gp120 shedding, as well as soluble CD4 [10,11].

Structure–activity analysis of these CD4 CDR3-related peptides showed that a scrambled peptide, S1, altered in the amino acid sequence of the CD4 CDR3-related peptide CD4(66–92) but not in overall composition, has stronger anti-HIV-1 activity than the original native sequence [12]. In this study, we examined the effect of the S1 peptide upon anti-HIV-1 activities after further modification, including D-isomerization, dimerization and conjugation with lipophilic substances such as phospholipids. The results indicated that the dimer derivative had increased activities at micromolar concentrations.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; CDR, complementarity-determining region; CD, circular dichroism; TCID, tissue culture infectious dose; PE, phosphatidylethanolamine; CPE, cytopathic effect.

2. MATERIALS AND METHODS

2.1. *Synthesis of CD4 CDR3-related peptides*

CD4 CDR3-related peptides (Table I) were synthesized on a Milli-Gen/Biosearch 9050 peptide synthesizer using Fmoc chemistry [13]. The peptides were purified by reverse-phase HPLC (> 95% purity), then characterized by amino acid analysis and fast atom bombardment mass spectrometry: CD4(66–92), *m/z* 3,316.32 (calculated 3,316.74); S1, *m/z* 3,316.79; S1-D, *m/z* 3,316.46; CS1, *m/z* 3,419.33 (calculated 3,419.78); SS1-C, *m/z* 2,257.71 (calculated 2,257.64). Lyophilized peptides were stored desiccated at − 20°C. The homodimer peptide (CS1 (dimer)) was prepared by air-oxidizing the CS1 (S1 analogue containing N-terminal cysteine) in 50 mM sodium phosphate buffer (pH 7.2) at a concentration of 2 mg/ml for 6 h at room temperature. HPLC analysis confirmed the quantitative formation of the dimer peptide. The peptide–phospholipid conjugate (S1-PE) was prepared by derivatizing egg yolk phosphatidylethanolamine (PE) with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) [14] and coupling the thiolated PE with CS1 at a molar ratio of 1:1.

2.2. *Cells and viruses*

M10 [15], a clonal cell line of MT-4, and MOLT-4 clone No. 8 [16] cells were maintained in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, as described previously [12]. The conditioned medium of MOLT-4 cells persistently infected with $HIV\text{-}1_{HTLV\text{-}IIIB}$ (MOLT-4/HIV-1) was used as a virus inoculum.

2.3. *HIV-1 infectivity assay*

The ability of CD4 CDR3-related peptides to reduce viral infectivity ($TCID_{50}$/ml) was tested as described previously [9]. Briefly, M10 cells in 96-well microplates (5×10^4 cells/well) were cultured with serial 10-fold dilutions of an HIV-1 inoculum which had been incubated with peptides for 30 min at 37°C. To determine the dose-dependence of the drugs, M10 cells were infected with HIV-1 (5×10^3 $TCID_{50}$) after an incubation with serial 4-fold dilutions of peptides for 30 min at 37°C. After incubation for 4 days at 37°C, the cells were fixed with cold acetone. HIV-1 antigen expression in the cells was detected by indirect immunofluorescence using serum from an HIV-1-seropositive patient.

2.4. *Cytopathic effect (CPE) assay without preincubation of HIV-1 with peptides*

M10 cells in 96-well microplates were mixed with HIV-1 (100 $TCID_{50}$) and CD4 CDR3-related peptides in duplicate (2.5×10^4 cells/well, 100 μl), then incubated for 6 days at 37°C. On day 3, 100 μl of fresh medium containing appropriate concentrations of peptides was added to each well. The ability of the peptides to inhibit virus-induced CPE in M10 cells was evaluated by the viable cell number determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [17].

2.5. *Assay of HIV-1-induced syncytium formation*

MOLT-4/HIV-1 cells (5×10^3) in 96-well microplates were incubated with CD4 CDR3-related peptides for 30 min at 37°C, then co-cultured with MOLT-4 clone No. 8 cells (5×10^4) for 24 h at 37°C in triplicate, as described [12]. The number of syncytia was counted microscopically and the relative syncytium formation (%) to the untreated control was calculated.

2.6. *Cytotoxicity assay*

Cells in 96-well microplates (5×10^4 cells/well) were incubated in the presence or absence of CD4 CDR3-related peptides for 3 days at 37°C in duplicate, and the cell viability was determined by the MTT method.

2.7. *Assay of p24 antigen capture*

The amount of p24 antigen in the conditioned media of MOLT-4/HIV-1 cells was determined using a commercial ELISA kit.

2.8. *Circular dichroism (CD) analysis*

CD spectra of CD4 CDR3-related peptides at ≈ 0.1 mg/ml in 10 mM sodium phosphate buffer, pH 7.2, were measured in a 0.2 cm- pathlength cell on a Jasco J-720 spectropolarimeter. Temperature scans were taken at a slope of 50°C/h. Secondary structure contents were estimated using the program, J-700/98 SSE-338 Ver. 1.00e (Jasco), as described [12].

2.9. *Chemical reagents*

Reagents were obtained commercially: Fmoc-D/L-amino acids from Watanabe Chemical Industries (Hiroshima, Japan); egg yolk PE and MTT from Sigma (St. Louis, MO); SPDP from Pierce (Rockford, IL); FITC-conjugated rabbit anti-human IgG from Dakopatts A/S (Copenhagen, Denmark); p24 antigen capture ELISA kit from American Bio-Technologies (Cambridge, MA).

3. RESULTS

3.1. *Anti-HIV-1 activity of S1-derived peptides*

CD4 CDR3-related peptide derivatives were first screened for their inhibitory effect on HIV-1 infectivity by measuring residual viral infectivity after incubating the HIV-1 inoculum with the peptides for 30 min at 37°C (Table II). Both S1-D (D-isomer of S1) and S1 (L-isomer) showed strong inhibitory activity against HIV-1. In addition, air-oxidized CS1 (dimer) and S1-PE (phospholipid conjugate of CS1), completely blocked HIV-1 infectivity in this assay system. SS1-C, which contains about two-thirds of the C-terminal portion of S1 and the N-terminal serine, was less active.

Table I

Amino acid sequences of the CD4 CDR3-related peptides studied

Peptide	Sequence
CD4(66–92)	NFPLIIKNLKIEDSDTYICbEVEDQKEE
S1	DTSEDEVIEKILKNCbKIQIELDPYFEN
S1-D	**DTSEDEVIEKILKN**Cb**KIQIELDPYFE**N
CS1	CDTSEDEVIEKILKNCbKIQIELDPYFEN
SS1-C	SILKNCbKIQIELDPYFEN

Bold residues represent D-amino acids. Cb, *S*-benzyl-L-cysteine.

Table II

Inhibitory effect of CD4 CDR3-related peptides on HIV-1 infectivity

Peptide	Conc. (mg/ml)	$TCID_{50}$/ml[a]
No peptide		$10^{5.5}$
CD4(66–92)	1	$10^{3.5}$
S1	1	$10^{2.0}$
S1-D	1	$10^{1.66}$
CS1 (dimer)	1	$<10^{1.5}$
S1-PE	0.25	$<10^{1.5}$
SS1-C	0.5	$10^{4.5}$

[a] HIV-1 was incubated for 30 min with indicated concentrations of peptides at 37°C, and residual infectivity ($TCID_{50}$/ml) was determined in M10 cells.

We then examined the dose-dependence and cytotoxicity of the peptides. As shown in Fig. 1, S1-D was as potent as S1, and CS1 (dimer) was 10- to 15-fold more active than S1 in inhibiting viral infection in M10 cells, as evidenced by immunofluorescence. The doses required for a 50% reduction of viral antigen expression (IC_{50}) were 205 and 17 μg/ml for S1-D and CS1 (dimer), respectively (Table III). When cells were infected with HIV-1 without preincubating the virus with peptides, slightly higher doses were required for similar inhibition in the CPE assay; the IC_{50} were 450 and 40 μg/ml for S1-D and CS1 (dimer), respectively (Table III). Under the same assay conditions, S1-PE had an IC_{50} of 25 μg/ml, although toxicity to M10 cells appeared, namely, a CC_{50} (dose for 50% cytotoxicity) of 240 μg/ml. Other peptides showed no cytotoxicity even at 1,00 μg/ml.

The peptides also inhibited HIV-1-induced syncytium formation. Under our assay conditions, MOLT-4/HIV-1 cells were first incubated with the peptides for 30 min at 37°C, then co-cultured with uninfected MOLT-4 clone No. 8 cells. The inhibition of syncytium formation between the infected and uninfected T cells by S1-derived peptides was dose-dependent. The IC_{50} values were 80, 56 and 4.4 μg/ml for S1, S1-D and CS1 (dimer), respectively (Table III).

3.2. *Effect of CD4-related peptides on HIV-1 production by persistently HIV-1-infected cells*

CD4(66–92), S1 and S1-derived peptides were examined for their inhibitory effects on the HIV-1 production from MOLT-4/HIV-1 cells. MOLT-4/HIV-1 cells at 5×10^5/ml were cultured for 3 days in the presence of the peptides. None of the peptides were cytotoxic (Fig. 2). However, the ELISA assay of p24 antigen in the conditioned medium revealed no significant effect of the peptides upon HIV-1 production. This indicates that these

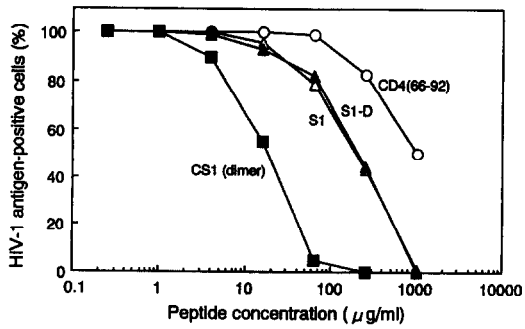

Fig. 1. Dose-dependent inhibitory effect of S1-derived peptides on the expression of HIV-1-specific antigen in M10 cells. M10 cells (5×10^4) were cultured with HIV-1 (5×10^3 $TCID_{50}$) which had been preincubated with CD4 CDR3-related peptides for 30 min at 37°C. After 4 days, HIV-1 antigen-positive cells were identified by indirect immunofluorescence.

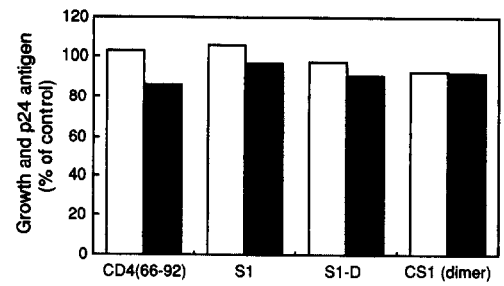

Fig. 2. Effect of CD4 CDR3-related peptides on persistently HIV-1-infected cells. MOLT-4/HIV-1 cells were incubated without or with the peptides at 1 mg/ml for 3 days at 37°C. Cell growth (open bars) was determined by the MTT method, while HIV-1 p24 antigen in the conditioned medium (closed bars) was measured by ELISA.

peptides block HIV-1 infectivity and syncytium formation but lack the ability to prevent viral replication in, or release from, already infected cells.

3.3. *Structure analysis of CD4-related peptides by CD*

The structures of S1-derived peptides in solution were analyzed by CD. Fig. 3A shows a comparison of the ultra-violet CD spectra of S1, S1-D and CS1 (dimer) in 10 mM phosphate buffer, pH 7.2, at 37°C. The CD spectrum of S1-D was the mirror image of that of S1, indicating that S1-D fully retains the D-amino acid configuration in solution. The CD spectrum of CS1 (dimer) was similar to that of S1, but the negative CD intensity at a shoulder peak around 218–222 nm was stronger in CS1 than in S1. The CD spectrum of CS1 (dimer) exhibited another negative peak at 206 nm and a strong positive peak at 192 nm. These results are indicative of helix–helix interactions [18] in the dimer CS1. We then examined the effect of temperature on the CD intensity at 220 nm, at which α-helix-to-random coil transitions are prominent [19]. Fig. 3B shows thermal unfolding profiles of S1 and CS1 (dimer) measured by CD at 220 nm. The negative CD intensity of the dimer CS1 was progressively reduced with increasing temperature, whereas no apparent change in S1, coinciding with a previous observation that β-sheets or random coils without substantial helix conformations, were detected in S1 in solution [12]. These results indicate the presence of a temperature-sensitive secondary structure, that is, an α-helix in the disulfide-crosslinked CS1, but not in S1. The estimated helical contents of dimer CS1 and S1 (11.8% and 2.0%, respectively), from their CD spectra based on the method of Yang et al. [20], support this conclusion. Thus, it is noteworthy that the structure of dimer CS1 at 37°C could be stabilized by this α-helix formation, contributing to its increased anti-HIV-1 activities.

4. DISCUSSION

The D-isomer of S1 (S1-D), fully retaining D-amino acid configuration in solution, was as potent as S1 in inhibiting HIV-1 infection and HIV-1-induced syncytium formation. This suggests that there are no configurational restraints upon its antiviral activity.

Table III

Summary of anti-HIV-1 activities and cytotoxicity of CD4 CDR3-related peptides

Peptide	Free virus infection			Syncytium formation	
	IC_{50} (μg/ml)		CC_{50} (μg/ml) (M10)	IC_{50} (μg/ml)	CC_{50} (μg/ml) (MOLT-4)
	Preincubation[a]	No preincubation			
CD4(66–92)	990	> 1,000	1,000	340	1,000
S1	190	300	1,000	80	1,000
S1-D	205	450	1,000	56	1,000
CS1 (dimer)	17	40	1,000	4.4	1,000
S1-PE	ND[b]	25	240	ND	ND

[a] HIV-1 was incubated with peptides for 30 min at 37°C before being added to M10 cells.
[b] ND, not determined.

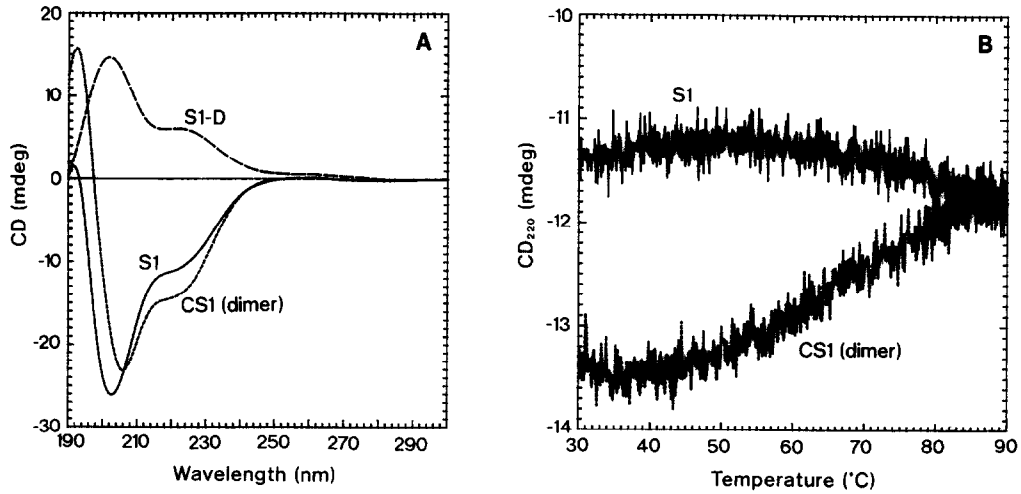

Fig. 3. CD spectra of CD4 CDR3-related peptides. (A) The CD spectra of S1, S1-D and CS1 (dimer) at 37°C. CD intensity is given in millidegrees (mdeg). (B) The effect of temperature on the CD intensity of S1 and CS1 (dimer) measured at 220 nm.

Several groups have reported that CD4 CDR3-related peptide derivatives and polyanionic compounds, such as sulfated polysaccharides, specifically bind to the gp120 V3 loop [21–23]. We also found that S1 inhibits the binding of anti-V3 monoclonal antibodies to the V3 loop, even at a lower concentration than that showing antiviral activities [11]. Acidic amino acid residues are clustered at the N-terminus portion in S1, while the dispersed form of the acidic residues has greatly reduced antiviral activities [12]. The V3 loop of gp120 contains a high concentration of positively charged residues which are highly conserved among divergent HIV-1 isolates [22,24]. Therefore, the anti-HIV-1 activities of S1 and S1-derived peptides could be the result of direct electrostatic interactions between the polyanionic region in the peptides and the cationic cluster present in the V3 loop.

Another important finding in the present study was the increased anti-HIV-1 activity of the dimer analogue of S1. The homodimer CS1 peptide exerted antiviral activities with an IC_{50} of 4.4–17 μg/ml (0.6–2.5 μM) when preincubated with HIV-1 or HIV-1-infected cells before contact with uninfected cells (Table III). CD analysis (Fig. 3A) indicated that an α-helical conformation was present in the secondary structure of the dimer peptide at a ratio of 12% at 37°C. No thermal unfolding was detected in the dimer peptide at temperatures below 40°C (Fig. 3B). These results suggest that this modification stabilized the secondary structure of the peptide by α-helix formation, probably enabling the stable interaction with the viral envelope glycoprotein. These results appear consistent with the previous observation that disulfide bridging of a synthetic peptide derived from gp41 (amino acid residues 558–595) stabilized the peptide structure and increased its antiviral activity [25]. Thus, the increased activities of CD4 CDR3-related peptide derivatives examined here may provide an alternative anti-HIV-1 approach by targeting the gp120 V3 loop.

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